EPA/OPP MICROBIOLOGY LABORATORY
ESC, Ft. Meade, MD

Standard Operating Procedure
for
AOAC Use Dilution Method for Testing Disinfectants

SOP Number: MB-05-03

Date Revised: 09-10-02

Prepared By: _____Date: ___/___/___

Print Name:_____

Reviewed By: _____ Date: ___/___/___

Print Name:_____
Technical Staff

_____ Date: ___/___/___

Print Name:_____
QA Officer

_____ Date: ___/___/___

Print Name:_____
Laboratory Director

Date Issued: ___/___/___

Withdrawn By: _____ Date: ___/___/___

Controlled Copy No.: _____

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1.0 SCOPE AND APPLICATION:

1.1 This SOP describes the methodology used to determine the efficacy of disinfectants against two organisms, *Pseudomonas aeruginosa and Staphylococcus aureus*.

2.0 DEFINITIONS:

2.1 API = Analytical Profile Index

2.2 AOAC = AOAC International

2.3 OD = outside diameter

2.4 ID = inside diameter

3.0 HEALTH AND SAFETY:

3.1 All manipulations of the test organism are required to be performed in accordance to biosafety practices stipulated in SOP MB-01, Lab Biosafety.

3.2 Disinfectants may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, phenol, etc. Latex gloves and other personal protective clothing or devises are worn during the handling of these items for purpose of activation, dilution, or efficacy testing. A chemical fume hood or other containment equipment is employed when performing tasks with concentrated products.

4.0 CAUTIONS:

4.1 Follow appropriate chain-of-custody guidelines during testing as stipulated in SOP-COC-01, Sample Log-in and Tracking.

4.2 Strict adherence to the protocol is necessary for the validity of the test results.

5.0 INTERFERENCES: None

6.0 PERSONNEL QUALIFICATIONS:

6.1 Personnel are required to be knowledgeable of the procedures in this SOP. Documentation of training and familiarization with this SOP can be found in the training file for each employee.

7.0 SPECIAL APPARATUS AND MATERIALS:

7.1 *Pseudomonas aeruginosa* (ATCC #15442)

7.2 *Staphylococcus aureus* (ATCC #6538)

7.3 Carriers: polished stainless steel cylinders, 8 +/- 1 mm OD, 6 +/- mm ID, 10 +/- 1mm length; type 304 stainless steel, SS 18-8 (S & L Metal Products Corp., Maspeth, NY)

8.0 INSTRUMENT OR METHOD CALIBRATION:

8.1 Refer to the laboratory equipment calibration and maintenance SOPs (SOP EQ series) for details on method and frequency of calibration.

9.0 SAMPLE HANDLING AND STORAGE:

9.1 Disinfectants are stored according to manufacturers' recommendations or at room temperature if the product label or testing parameters do not identify a storage temperature. Those disinfectants requiring activation or dilution prior to use will only be activated or diluted within three hours of testing unless test parameters specify otherwise.

10.0 PROCEDURE AND ANALYSIS:

10.1 Brief Summary: The AOAC Use-dilution test is a carrier-based test. Carriers (stainless steel cylinders) are inoculated with a test organism, dried, exposed to the use-dilution of the disinfectant product, and cultured to assess the survival of the bacteria. A single test involves the evaluation of 60 inoculated carriers (one organism) against one product sample. In addition to the 60 carriers, 6 carriers are required to estimate carrier bacterial load and 6 more are included as extras. Thus, a total of 72 seeded carriers are required to perform a single test.

10.2 Test Culture Preparation:

10.2.1 Initiate test culture by inoculating a 10 mL tube (20 mm x 150 mm) of nutrient broth or synthetic broth from a stock slant or stab culture. Transfer a loopful of inoculum from the stock culture into the broth. Refer to SOP MB-02, Test Microbes: Initiation, Maintenance and Quality Control, for stock culture preparation.

10.2.2 Two sets of cultures (one set as a backup) of the same organism may be initiated in parallel from the same stock culture and subcultured; however, only one set of the final cultures is used for actual testing. Select set with typical growth.

10.2.3 The test culture is serially subcultured for at least three consecutive 24±2 hour periods in 10 mL of nutrient broth or synthetic broth at 37±1°C.

10.2.4 Following the three consecutive subcultures, the test culture is subcultured once again in nutrient broth or synthetic broth. For this final subculture step, inoculate eight 25 mm × 150 mm tubes containing 20 mL of nutrient broth or synthetic broth for each test of *S. aureus*. Inoculate ten tubes of nutrient broth or synthetic broth for each test of *P. aeruginosa*. Incubate at 37±1°C for 48 to 54 hours.

10.2.5 Five days, minimum, are required to obtain the culture for seeding carriers. For example, the culture sequence must begin on Thursday for testing to commence on the following Tuesday.

10.2.6 Record all culture transfers on the Organism Culture Tracking form (see SOP QC-15, Media Prep and Sterilization Run Numbers).

10.3 Carrier Inoculation:

10.3.1 Pool the eight or ten 48-54 hour test cultures in a sterile

flask. The pellicle from the 48-54 hr *Pseudomonas* cultures must be removed from the broth before pooling the culture either by decanting the liquid culture aseptically into a sterile tube or by gently aspirating the broth culture away from the pellicle using a sterile 10 mL pipette. In either case, the pellicle must not be broken or fragmented or the culture is not usable. Swirl to mix.

10.3.2 If no organic soil is required, aliquot 20 mL portions of the culture into sterile 25 mm x 150 mm test tubes. If an organic soil load is to be added to the culture, measure the pooled culture (or the remaining pooled culture) and add the appropriate amount of soil to the flask. Swirl to mix. Aliquot 20 mL portions into sterile 25 mm x 150 mm test tubes.

10.3.3 Vortex the 20 mL cultures for 3-4 sec and let stand 10 minutes at room temperature.

10.3.4 Withdraw the top 3/4 of the culture from each tube with a sterile pipette and dispense a total of 20 mL into sterile 25 mm x 150 mm test tubes. Cultures may be combined from more than one tube to achieve the 20 mL total. Prepare four tubes in this way.

10.3.5 Using a sterile hook, aseptically transfer twenty carriers from the asparagine storage solution (see SOP MB-03, Screening Carriers) into each of the four tubes containing 20 mL of test culture. Drain the asparagine solution from the cylinders by tapping them against the side of the tube before transferring. Use only carriers that have been physically screened and have passed bioscreening (see SOP MB-03, Screening Carriers).

10.3.6 Three to four carriers may be transferred on a single hook. The hook does not need to be flamed for each transfer. The test culture must completely cover the carriers. If a carrier is uncovered, gently shake the tube, or reposition the carrier within the tube with a sterile hook.

10.3.7 After 15 minutes of contact time with the broth culture, remove the carriers using a sterile wire hook. Tap the carriers vigorously against the side of the tube to remove any excess culture.

10.3.8 Place each carrier on its end in a sterile glass petri dish containing a double layer of sterile Whatman No. 2 filter paper.

10.3.9 Transfer twelve carriers from the culture tubes to each of 6 petri dishes. Place lid on petri dish when complete.

10.3.10 The carriers can be removed from the test culture by transferring more than one on each hook. Once the carriers are deposited in the petri dish, they cannot touch each other or tip over. Those that do cannot be used and must be removed and recleaned.

10.3.11 Once all (72) of the carriers have been transferred, dry for 40 minutes in a 37±1°C incubator. Following the dry time, the seeded carriers can be used for testing. The seeded carriers must be used the day of preparation.

10.3.12 Record the timed carrier inoculation activities on the Time Recording Sheet for Carrier Inoculation Steps (see 16.1).

10.4 Disinfectant Sample Preparation:

10.4.1 Turn on the recirculating chiller and allow it to come to 20±1°C or the temperature specified by the disinfectant manufacturer.

10.4.2 Ready-to-use products are tested as received; no dilution is required.

10.4.3 Prepare disinfectant samples aseptically according to the manufacturers' instructions. Use dilution of the product, contact time, temperature, diluent, organic soil, hard water, and neutralizers are identified in the test parameters.

Record test parameter information on the Test Information Sheet (see 16.3).

10.4.4 To ensure stability, prepare the disinfectant dilutions within three hours of performing the assay unless test parameters specify otherwise.

10.4.5 Prepare all dilutions with sterile standardized volumetric glassware.

10.4.6 Prior to opening the container of a liquid product, gently shake the container and thoroughly clean the area around the cap and spout with 70% ethanol. Allow the surface to dry. Remove the cap. Do not touch the inside surface of the cap. If present, carefully remove the seal attached to the lip of the spout with cooled, flame-sterilized instruments (i.e., razor blade, forceps).

10.4.7 Pour an appropriate aliquot of the sample into a sterile beaker. Do not place a pipette or any other instrument inside the product container. Place cap on the product container and secure tightly. From the beaker, dispense ready-to-use products directly into sterile medication tubes or initiate dilutions for diluted products.

10.4.8 For diluted products, use $\geq$ 1.0 mL of sample disinfectant to prepare the use-dilution to be tested. Use v/v dilutions for liquid products and w/v dilutions for solids. Round to two decimal places toward a stronger product. Record disinfectant preparation on the Media/Reagent Preparation Sheet (see SOP QC-15, Media Prep and Sterilization Run Numbers). For one Use-dilution test, prepare approximately 1 L of the disinfectant.

10.4.9 Dispense 10 mL aliquots of the diluted disinfectant or ready to use product into seventy 25 mm x 100 mm medication tubes (60 for testing and 10 extra).

10.4.10 Place medication tubes in the water bath for approximately

10 minutes.

10.5 Test Procedure:

10.5.1 After the required drying time, the carriers are sequentially transferred at either 20 sec or 30 sec intervals from the petri dish to the medication tubes containing the disinfectant using a sterile hook. Modify these intervals to accommodate exposure times other than 10 min.

10.5.2 One carrier is added per tube. Immediately after placing carrier in the medication tube, swirl 3 times before placing it back in the bath. The carrier must be deposited in the tube within ±5 sec of the prescribed drop time. Flame the hook and allow it to cool after each carrier transfer.

10.5.3 Reminder: When lowering the carriers into the medication tubes, neither the carrier itself nor tip of the wire hook can touch the interior sides of the tube. If the interior sides are touched, the tube number is noted on the AOAC Use-Dilution Test Results Sheet (see 16.0). If the cylinder yields a positive result, it is not counted.

10.5.4 After one set of carriers has been deposited into the disinfectant, and the exposure time is complete, the carriers are then transferred with the same sequential timed fashion into the primary subculture tubes (see 10.5.1) containing the appropriate neutralizer (10 mL in 20 mm x 150 mm tubes). The carrier is removed from the medication tube with a sterile hook, tapped against the interior sides of the tube to remove the excess disinfectant, and transferred into the primary subculture tube.

10.5.5 Flame hook after each carrier transfer.

10.5.6 The remaining carriers are moved into their corresponding subculture tubes at the appropriate time. The carrier can touch the interior sides of the subculture tube during the transfer, but contact should be avoided as much as possible.

10.5.7 After the carrier is deposited, the subculture tube is recapped and vortexed for 3-4 seconds. Alternately, the tubes may be vortexed after all primary transfers are completed. As with the transfers to the medication tubes, transfers into primary subculture tubes should be within ±5 sec of the actual time of transfer.

10.5.8 Record timed events on the Time Recording Sheet for Carrier Transfer Form (see 16.2).

10.5.9 The bacterial carrier load on six carriers is assayed as stipulated in SOP MB-04, Carrier Counts.

10.5.10 After all the carriers have been transferred and vortexed, the subculture tubes are placed in a 37±1°C incubator.

10.5.11 After a minimum of 30 minutes from when the last carrier was deposited, transfer the carriers using a sterile wire hook to a second subculture tube containing 10 mL of the appropriate neutralizer. Sixty secondary tubes are required per test, one for each carrier. Move the carriers in order but the movements do not have to be timed.

10.5.12 Vortex the tubes after all of the carriers have been transferred. Both the primary and secondary subculture tubes are placed back into the 37°C incubator, and incubated for at least 48±2 hours. Read the results after this time.

10.5.13 See Attachment A (Testing Footnotes and Explanations) for a list of footnotes which are used to indicate problematic events or observations which occurred during testing.

10.6 Results:

10.6.1 Report results as + (growth) or 0 (no growth) on the AOAC Use-dilution Results Sheet (see 16.4). A positive result is one in which the broth culture appears turbid. A negative result is one in which the broth appears clear. Each tube is

shaken prior to recording results to determine the presence or absence of turbidity. The primary and secondary subculture tubes for each carrier represent a "carrier set".

10.6.2 A positive result in either the primary or secondary subculture tube is considered a positive result for a carrier set.

10.7 Confirmation Steps:

10.7.1 A minimum of three positive carrier sets per test, if available, should be confirmed using gram staining, selective media and VITEK or API analysis. If there are less than three positive carrier sets, then each carrier set will be confirmed. If both tubes are positive in a carrier set, only one tube is selected for confirmatory testing.

10.7.2 For a test with greater than 20 positive carrier sets, confirm at least 20% by gram stain, and a minimum of 4 positive carrier sets by gram staining, selective media, and API or VITEK analysis (see SOP QC-16, VITEK: Culture Identification Numbers) to ensure the identity of the organism. Again, if both tubes are positive in a carrier set, only one tube is selected for confirmatory testing.

10.7.3 Gram stain reactions, cell morphology, and colony characteristics on selective media are given in SOP MB-02, Test Microbes: Initiation, Maintenance and Quality Control.

10.7.4 Gram stains are performed on smears taken from the positive culture tubes. For the additional confirmatory tests, a loopful of broth from each selected culture tube is streaked on both TSA and selective media appropriate for the test organism and incubated for 24±2 hr at 37±1°C. The selective agar is checked for the correct reaction and the culture on the TSA plate is used for preparing the inoculum for the API strips or the VITEK cards.

10.7.5 The API test should be performed according to the

manufacturer's instructions. The VITEK analysis should be performed according to the manufacturer's instructions.

10.7.6 If confirmatory testing determines that the identity of the organism was not the test organism, the positive entry (+) on the results sheet must be annotated to indicate a contaminant was present.

10.8 Re-use of Stainless Steel Carriers

10.8.1 When the test results are negative, the carriers may be reused after cleaning. Carriers that are positive are autoclaved, recleaned and screened biologically (see SOP MB-03, Screening Carriers). These carriers may be reused if the screening test results in no growth. The extra carriers that were inoculated but not used are autoclaved, recleaned and used again.

11.0 DATA ANALYSIS/CALCULATIONS: None

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

12.1 Data will be recorded promptly, legibly, and in indelible ink on the appropriate forms (see 16.0). Completed forms are archived in notebooks kept in locked file cabinets in file room D217. Only authorized personnel have access to the locked files. Archived data is subject to OPP's official retention schedule contained in SOP ADM-03, Records and Archives.

13.0 QUALITY CONTROL:

13.1 The OPP Microbiology Laboratory conforms to 40CFR Part 160, Good Laboratory Practices. Appropriate quality control measures are integrated into each SOP.

13.2 For quality control purposes, the required information is documented on the appropriate form(s) (see 16.0).

14.0 NONCONFORMANCE AND CORRECTIVE ACTION:

14.1 Strict adherence to the protocol is necessary for the validity of the test results. Any deviation from the standard protocols must be recorded on the form and an explanation for the deviation given.

15.0 REFERENCES:

15.1 Official Methods of Analysis. 1990. 15th Ed., Association of Official Analytical Chemists, Arlington, VA, (Method 955.14, 955.15, and 964.02).

16.0 FORMS AND DATA SHEETS:

16.1 AOAC Use-Dilution Test: Time Recording Sheet for Carrier Inoculation Steps

16.2 AOAC Use-Dilution Test: Time Recording Sheet for Carrier Transfers

16.3 AOAC Use-Dilution Test Information Sheet

16.4 AOAC Use-Dilution Test Results Sheet

Attachment A: Testing Footnotes and Explanations

AOAC Use-Dilution Test: Time Recording Sheet for Carrier Inoculation Steps

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Test Date	
Type of Test	
Product Reg. No.	
Product Name	
Sample No(s).	
Organism	

Initials/Date	Test ID	Inoculum Settle Time*		Carrier Seeding Time*		Carrier Dry Time*	
		Start Time	End Time	Start Time	End Time	Start Time	End Time
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/

* Recorded from laboratory clock/and timer.

AOAC Use-Dilution Test: Time Recording Sheet for Carrier Transfers

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Test Date	
Type of Test	
Product Reg. No.	
Product Name	
Sample No(s).	
Organism	

Initials/date	Set	Drop Interval	Carrier Drop Start Time (into the disinfectant)		Carrier Drop End Time (into the primary subculture/neutralizer media)		Carrier Transfer (into secondary subculture)
			Clock	Timer	Clock	Timer	Start Time[1]
	1-20						
	21-40						
	41-60						
Comments:							

1 Carrier transfer into secondary subculture (time elapsed after last carrier dropped in primary); taken from clock

AOAC Use-Dilution Test Information Sheet

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		SOP	
Name		Test Date	
Sample No.		Comments:	
Lot No.			

TEST PARAMETERS/Confirmed by:_____			
H_2O Hardness ($CaCO_3$) ppm	Specified	Titrated (Buret)/Date/Init.	HACH/Date/Init.
		/ /	/ /
Use Dilution	Specified	As Prepared/Date/Init.	
		/ /	
Organic Soil	Specified	As Prepared/Date/Init.	
		/ /	
Neutralizer	Specified		
Temperature	Specified	Chiller Unit Display	Test Tube Water Bath
		Before: After:	Before: After:
Contact Time	Specified	As Tested	
Other Parameters	Specified		

TEST MICROBE INFORMATION/Confirmed by:_____				
Test Microbe		48-54 Hour Culture		
Org. Control No.		Date/Time	Initiated	Harvested
Avg. CFU/Carrier				

REAGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.

AOAC Use-Dilution Test Results Sheet
OPP Microbiology Laboratory

PRODUCT INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Name		Test Organism	

CARRIER INFORMATION (to be completed by Analyst)		
Carrier Drop Time Interval	Carrier Set	Analyst

TEST RESULTS									
Date Recorded/Initials									
Primary Subculture / Secondary Subculture (carrier)									
1	2	3	4	5	6	7	8	9	10
/	/	/	/	/	/	/	/	/	/
11	12	13	14	15	16	17	18	19	20
/	/	/	/	/	/	/	/	/	/
21	22	23	24	25	26	27	28	29	30
/	/	/	/	/	/	/	/	/	/
31	32	33	34	35	36	37	38	39	40
/	/	/	/	/	/	/	/	/	/
41	42	43	44	45	46	47	48	49	50
/	/	/	/	/	/	/	/	/	/
51	52	53	54	55	56	57	58	59	60
/	/	/	/	/	/	/	/	/	/
Results Summary			Number of Carrier Sets with Growth						
			Number of Carrier Sets without Growth						
Modifications/Comments:									

Attachment A:

Testing Footnotes and Explanations
OPP/Microbiology Laboratory

Footnote	
A	Indicates that the seeded carrier, hook, or forceps hit the interior sides of the medication tube containing disinfectant as the carrier was being dropped.
B	Indicates that the carrier was lost (dropped) during a transfer and was not recovered.
C	Indicates that a tube of a positive carrier set (one showing growth) was later determined to be a contaminant and not the test microbe. In "Comments" refer to the confirmation information for details.
D	Indicates that the primary or secondary subculture tube containing the carrier broke during vortexing. In the "Comments" indicate if carrier was recovered or if the remaining broth was placed in another tube.
E	Indicates that the carrier was exposed to the disinfectant late or early, outside of the +/- 5 second drop, spray, or wipe interval. In "Comments" indicate the approximate number of seconds outside (+/-) of the 5 second interval.
	Indicates that the carrier was placed in the neutralizer late or early, outside of the +/- 5 second drop interval. In "Comments" indicate the approximate number of seconds outside (+/-) of the 5 second interval.